The role of Cyclin D1 and Ki-67 in the development and prognostication of thin melanoma

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Abstract: Background Despite their low individual metastatic potential, thin melanomas (1 mm Breslow thickness) contribute significantly to melanoma mortality overall. Therefore, identification of prognostic biomarkers is particularly important in this subgroup of melanoma. Prompted by pre-clinical results, we investigated cyclin D1 protein and Ki-67 expression in in situ, metastatic and non-metastatic thin melanomas. Material and Methods Immunohistochemistry was performed on 112 melanoma specimens, thereof 22 in situ, 48 non-metastatic and 42 metastatic thin melanomas. Overall, epidermal and dermal cyclin D1 and Ki-67 expression were semi-quantitatively evaluated by three independent investigators and compared between groups. Results Epidermal Ki-67 expression did not differ statistically in in situ and invasive melanoma (P = 0.7). Epidermal cyclin D1 expression was significantly higher in thin invasive than in in situ melanoma (P = 0.003). No difference was found in cyclin D1 expression between metastatic and non-metastatic invasive tumours. Metastatic and non-metastatic thin melanomas did not show significant differences in epidermal expression of Ki-67 and cyclin D1 (P = 0.148 and P = 0.611, respectively). In contrast, strong dermal expression of Ki-67 was more frequent in metastatic than non-metastatic samples (28.6 vs. 8.3%, respectively, P = 0.001). The prognostic value of dermal Ki-67 expression was confirmed by multivariate analysis (P = 0.047). Conclusion We found an increased expression of cyclin D1 invasive thin melanomas compared to in situ melanomas which supports a potential role of this protein in early invasion in melanoma, as suggested by pre-clinical findings. Moreover, our results confirm that high dermal Ki-67 expression is associated with an increased risk of metastasis development in thin melanoma and could possibly serve as a prognostic biomarker in clinical practice, especially if combined with additional methods.

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The role of Cyclin D1 and Ki-67 in the development and prognostication of thin melanoma

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ABSTRACT

Background

Despite their low individual metastatic potential, thin melanomas (≤1 mm Breslow thickness) contribute significantly to melanoma mortality overall. Therefore, identification of prognostic biomarkers is particularly important in this subgroup of melanoma. Prompted by pre-clinical results, we investigated cyclin D1 protein and Ki-67 expression in in situ, metastatic and non-metastatic thin melanomas.

Material and Methods

Immunohistochemistry was performed on 112 melanoma specimens, thereof 22 in situ, 48 non-metastatic and 42 metastatic thin melanomas. Overall, epidermal and dermal cyclin D1 and Ki-67 expression were semi-quantitatively evaluated by three independent investigators and compared between groups.

Results

Epidermal Ki-67 expression did not differ statistically in in situ and invasive melanoma ($P = 0.7$). Epidermal cyclin D1 expression was significantly higher in thin invasive than in in situ melanoma ($P = 0.003$).

No difference was found in cyclin D1 expression between metastatic and non-metastatic invasive tumours. Metastatic and non-metastatic thin melanomas did not show significant differences in epidermal expression of Ki-67 and cyclin D1 ($P = 0.148$ and $P = 0.611$, respectively). In contrast, strong dermal expression of Ki-67 was more frequent in metastatic than non-metastatic samples (28.6 vs. 8.3%, respectively, $P = 0.001$). The prognostic value of dermal Ki-67 expression was confirmed by multivariate analysis ($P = 0.047$).

Conclusion

We found an increased expression of cyclin D1 invasive thin melanomas compared to in situ melanomas which supports a potential role of this protein in early invasion in melanoma, as suggested by pre-clinical findings. Moreover, our results confirm that high dermal Ki-67 expression is associated with an increased risk of metastasis development in thin melanoma and could possibly serve as a prognostic biomarker in clinical practice, especially if combined with additional methods.

INTRODUCTION

The incidence of invasive cutaneous melanoma has been steadily increasing in countries of the western world for more than five decades (1). Owing to improved skin cancer screening and diagnostic techniques, the majority of cutaneous primary melanomas is diagnosed at an in situ or early invasive stage. Despite the low individual metastatic potential of these tumours, the subgroup of thin invasive melanomas (up to 1 mm in Breslow thickness) accounts for the highest absolute number of melanoma-related deaths of all four T subcategories as defined by the American Joint Committee on Cancer (AJCC) staging system (2-4). Hence, understanding of the early cellular and genetic mechanisms that enable melanoma cells to breach the basement membrane and acquire invasive potential is crucial to allow for early identification of selected patients at increased risk of developing metastatic disease. In regard to genetics, melanoma development has been described as a distinct step-wise process with a successive evolution from unequivocally benign to intermediate, early malignant (in situ) and invasive malignant melanocytic lesions based on distinct alterations, including initial BRAF V600E mutations amongst others (5). The diverse cellular consequences of these oncogenic mutations that confer tumour growth, invasion and metastatic potential constitute the well-known hallmarks of cancer (6). One of these hallmarks is sustained proliferative signalling which in vivo can be depicted through detection of proliferation markers such as the Ki-67 protein. This protein is expressed in the nuclei of cells in the G1, S, and G2 phase of the cell cycle as well as in mitosis (7). The functional significance of the Ki-67 protein within the cell cycle remains to be fully elucidated. Important roles in cancer stem cell maintenance and organization of the chromosome periphery during mitosis have been described. Clinically, increased Ki-67 expression is a well-known marker of poor prognosis in various malignancies such as breast cancer (8). In melanoma, its prognostic role was most clearly shown in thick primary tumours (Breslow > 1 mm) (9, 10). In thin primary melanomas, particularly high dermal Ki-67 expression has been associated with increased risk for metastasis development (11).

Another potential marker of tumour cell proliferation is cyclin D1. This highly labile protein regulates G1/S transition in the cell cycle through interaction with cyclin-dependent kinases which mediate subsequent phosphorylation of the retinoblastoma protein leading

to increased proliferation amongst other cellular changes (12, 13). The CCND1 gene encoding for cyclin D1 is an established oncogene amplified in a variety of tumours such as breast, lung or endometrial cancer (12). Increased expression of cyclin D1 has also been reported in up to 62% of primary melanomas, while being minimal in melanocytic nevi (14). The oncogenic effect of cyclin D1 overexpression or gene amplification is primarily attributed to its impact on tumour cell proliferation, but also proliferation-independent oncogenic mechanisms have been proposed (12). These include effects of cyclin D1 on cellular migration and invasion, which were first described in macrophages (15). In this context, pre-clinical studies at our institution have shown that expression of cyclin D1 may confer early invasive properties of melanoma cells. As results concerning the prognostic significance of cyclin D1 expression in melanoma are inconsistent (16-19), based on our preclinical data, we hypothesized that cyclin D1 expression in primary melanoma may not merely be a surrogate for cellular proliferation, but also for early invasive properties of melanoma cells. Therefore, the present study investigated the expression and correlation of cyclin D1 and Ki-67 at different stages in the stepwise development of invasive melanoma. Both in situ and thin invasive primary melanomas were examined. Additionally, thin primary melanomas leading to the development of metastatic disease were specifically analysed in order to explore the potential role of cyclin D1 later in the process of invasion and metastasis development, hence re-evaluating its potential as a prognostic marker.

MATERIALS & METHODS

Patient selection and Data collection

The study was approved by both local ethics committees (Zurich [KEK-ZH-No. 647 and 800] and Salzburg [E-Nr. 2252]) prior to collection of data and tissue specimens. Clinical information was stored and analysed after encryption.

The study cohort consisted of patients with in situ (MIS), metastatic (MTM) and non-metastatic (NMTM) thin melanomas diagnosed in the period from 2008 and 2016 at two melanoma reference centres in Zurich and Salzburg. Disease stage was classified according to the 7th edition of AJCC staging system (20). Patients with multiple primaries, uveal or mucosal melanoma were excluded. Epidemiological, clinical and laboratory information were retrospectively collected from medical records. Multiple dermato-

pathological institutes in and around Zurich and an external collaborating pathology institute (Salzburg) served as sources for formalin-fixed paraffin-embedded (FFPE) samples of the respective primary tumours.

Immunohistochemistry (IHC)

Four different analyses (including H&E stain) were conducted on 3 µm thick FFPE-sections. Processing comprised of deparaffinization in xylene and rehydratation in decreasing ethanol concentrations, consecutive boiling for epitope retrieval in a 3-in-1 target retrieval solution (TRS6 or 9, Dako®, Agilent Technologies, Santa Clara, CA, USA) in a pressure cooker for a total of 40 minutes, followed by cooling for 20 minutes and rinsing with deionized water. Further staining was performed using the automated Dako® Autostainer Plus platform (see Supplementary Table 6). The following primary antibody clones were used: HMB-45 Clone HMB45 (M0634), Ki-67 Clone MIB 1 (M7240) and Cyclin D1 Clone EP12 (M3642), all manufactured by Dako®, Agilent Technologies, Santa Clara, CA, USA.

Immunoreactivity scoring

Three independent, blinded investigators (CK, PK and WK as a board certified dermatopathologist) performed semi-quantitative evaluation of all IHC stains resulting in a consensus-based score for each evaluation.

Based on previous studies (11), Ki-67 immunostaining was graded as low (≤20% positive neoplastic cells, score 1) and strong (≥20%, score 2). The consensus-based approach was particularly useful in tumours with a brisk immune infiltrate when distinction between Ki-67 positive dermal tumour cells and proliferating immune cells was necessary. This applied to 37 out of 90 primary tumours (41.1% of all invasive melanomas).

Cyclin D1 immunostaining was evaluated regarding expression intensity and frequency of positive tumour cells separately and combined using a modified Allred score method (21); scores (0) were graded as negative, (1-2) as weak, (3-5) as moderate, and (6-8) as strong. Additionally, cyclin D1 and Ki-67 expression were summed up as a composite score (1-4 low, 5-7 moderate, 8-10 strong). For further information, see Supplementary Table 1.

Statistical analysis

Excel 2016 (Microsoft Corporation, Redmond, WA, USA) and SPSS for Windows 25.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analysis. Statistical significance was defined by a two-sided *p*-value of less than 0.05. Chi-square test was applied for comparisons between groups (MIS vs. invasive melanoma, NMTM vs. MTM) concerning nominally scaled variables, i.e. IHC expression intensities and patterns. For analyses of small subgroups, Fisher's exact test was used, if applicable. For comparisons concerning metric variables, either the t-test or the Mann-Whitney-U-test were performed, depending on the assumed distribution of the respective variable. Multivariate analyses of potential prognostic factors were conducted through binary logistic regression. Pearson's bivariate analysis was conducted to assess potential correlations.

RESULTS

Data of 174 patients (65 MTM and 109 NMTM) was collected (see Supplementary Figure 1). Thereof, 52 (80%) and 94 (86.2%) blocks could be obtained, respectively. Final analysis comprised of 42 (80.8%) samples in the MTM and 48 (51.1%) samples in the NMTM group, which remained after exclusion due to discrepant Breslow thickness or insufficient tissue material. In addition, 22 MIS samples were included for comparison between MIS and invasive melanoma.

Patient and tumour characteristics

The study population consisted of 54 women and 58 men (see Table 1). Mean age at first diagnosis ranged from 49 to 72 years in the respective subgroups. The distribution of age between subgroups differed significantly ($P < 0.001$), as did the distribution of gender ($P = 0.032$). Median Breslow thickness was 0.78 mm for MTM and 0.59 mm for NMTM ($P < 0.001$). Ulceration status was known in 94% of tumours. There was no significant difference between frequency of ulceration in tumours of the MTM (5%) and the NMTM (10%) subgroup ($P = 0.45$).

The predominant histological subtype of invasive melanoma was superficial spreading melanoma (SSM) with 55 out of 112 samples (49.1%), representing 57.1% of the MTM and 43.8% of the NMTM group. In MIS patients, SSM and lentigo maligna melanoma (LMM) equalled with 45.5% (10 out of 22).

35.7% of all MTMs were localised on the trunk, whereas NMTMs were most frequently detected on the lower extremity (31.2%).

We did not detect a difference in mitotic rate (MR, documented as < 1 or ≥ 1 mitoses per mm^2) comparing invasive MTM and NMTM. Increased MR was found in 11 of 42 patients (26%) in the MTM and in 9 of 48 patients (19%, $P = 0.4$) in the NMTM group, respectively.

Sentinel lymph node biopsy (SLNB) was performed in a total of 22 out of 90 patients (24.4%) with invasive melanoma. SLNB was conducted in 38% of patients with MTM and 12.5% of patients with NMTM. In MTM patients, the SLNB result was positive in 9 patients (56%), while no patient in the NMTM group had a positive SLN (which would have been an exclusion criterion for this subgroup upfront).

Additional tumour characteristics of MTM and NMTM according to Breslow thickness can be found in Supplementary Tables 2-5.

Immunohistochemistry findings

The results of IHC findings in MIS, NMTM and MTM are summarized in Table 2.

First, epidermal expression of Ki-67 and cyclin D1 in MIS and invasive melanoma (MTM and NMTM grouped together) was analysed. No statistically significant difference in Ki-67 expression was observed between groups ($P = 0.7$). In contrast, a significantly higher percentage of cyclin D1 positive tumour cells was found in thin invasive melanoma compared to MIS ($P = 0.003$, Figure 1).

Second, epidermal and dermal expression in NMTM and MTM were compared. Epidermally, no difference was detected between both groups ($P = 0.15$ for Ki-67; $P = 0.61$ for cyclin D1). Cyclin D1 expression in the dermis was similar in both groups ($P = 0.88$). Contrarily, there was a significantly higher percentage of strong dermal Ki-67 expression in MTM compared to NMTM (28.6 and 8.3%; $P = 0.001$, Figure 2).

Analysing dermal Ki-67 expression together with the established prognostic parameter mitotic rate did not alter this finding significantly ($P = 0.003$). The prognostic value of epidermal Ki-67 expression was retained ($P = 0.047$) when other prognostic factors (Breslow thickness) and potential confounders (age, gender) – which were significantly different between groups – were taken into account in a multivariate analysis. There was

no correlation between Breslow thickness and dermal Ki-67 expression neither overall (Pearson's r = 0.17, *P* = 0.14), nor within the MTM subgroup (Pearson's r = -0.14, *P* = 0.46).

When analysing melanoma subtypes, epidermal cyclin D1 expression appeared to be increased in SSM compared to LMM in univariate analysis (*P* = 0.02). Also, there was a trend towards increased epidermal cyclin D1 expression in the small subgroup of acral lentiginous melanoma (ALM, 6 patients) compared to both SSM (*P* = 0.07) and LMM (*P* = 0.07), see Table 3.

Marginal note: Breslow measurement

Breslow thickness was re-measured for every sample obtained. As described above, 7 out of 174 specimens (4% of the total of MTM and NMTM) had to be excluded due initial underestimation of tumour thickness. Median mismeasurement was 0.75 mm (95% CI = 0.55 - 0.95).

In our study cohort (MTM and NMTM only, n = 90), documented Breslow was higher than the re-measured value in 32, lower in 36 and equal in 22 samples (35.6, 40 and 24.4%, respectively). Median discrepancy was 0.10 mm (95% CI = 0.07 - 0.13) and 0.14 mm (95% CI = 0.10 - 0.18).

DISCUSSION

Thin melanomas less than one millimetre in Breslow index do rarely metastasize, but, owing to their high incidence, still lead to a higher overall number of melanoma-related deaths than high-risk melanomas four millimetre or more in thickness (2, 3). The median duration between first melanoma diagnosis and death appears to be significantly longer in patients with thin primaries compared to those with thicker tumours (3). This may be explained by the more aggressive biology of thicker melanomas but may also result from later diagnosis of metastasis in patients with thin primaries. Late diagnosis of metastasis in turn could be the consequence of less stringent follow-up protocols in so-called low-risk patients with thin melanoma. Since more rigorous follow-up does not seem justifiable in this patient population, the discovery of biomarkers predictive of metastasis in thin melanoma is of utmost importance. The present study aimed to re-evaluate the predictive potential of two such potential biomarkers, namely Ki-67 and cyclin D1.

Our findings suggest that high dermal Ki-67 expression is an independent prognostic marker associated with an increased risk of metastasis formation in thin melanoma. This is in line with a large study by Gimotty et al. who also reported dermal, but not overall Ki-67 expression to be an independent prognostic marker in a cohort of 396 patients with more than 10 years of follow-up (11). Despite its significance, there are certain caveats for the use of Ki-67 in clinical practice. As mentioned in the methods section, it is frequently difficult to distinguish proliferating dermal tumour cells from proliferating immune cells in single antibody Ki-67 IHC, i.e. tumour infiltrating lymphocytes. Hence, double-staining IHC with a melanocytic differentiation marker such as Melan-A or MART-1 could increase sensitivity and specificity of Ki-67 staining. Combination with methods such as gene expression signatures could further augment prognostic value (22), albeit validity of such signatures remains to be confirmed in larger prospective studies.

Gimotty and others (11, 23-25) also reported on the prognostic potential of increased mitotic rate in thin melanoma, which previously was also included in the AJCC staging system (20). In our cohort of thin melanomas, we could not confirm the prognostic potential of mitotic rate, since increased mitoses were rare in both the metastatic and non-metastatic subgroup.

Investigating the stepwise development of melanoma from in situ to invasive lesions, we found statistically significant differences in epidermal cyclin D1 expression between in situ and invasive melanomas. These results contrast some studies on cyclin D1, which reported weak expression in more than two thirds of melanoma samples and no expression in nevi, respectively, but are in accordance with other reports describing overall high expression in melanocytic lesions (16, 26, 27). Ramirez et al. evaluated 126 pigmented skin lesions (thereof 28 in situ, 30 primary invasive, and 29 metastatic melanomas). They found a higher rate of cyclin D1 expression in invasive and in situ melanoma compared with benign melanocytic lesions. Contrary to our findings, these authors found an increased cyclin D1 expression in melanoma in situ compared to invasive melanoma, which could be explained by the higher number of melanomas over 1 mm Breslow thickness in this study, since the latter tumours showed a trend towards decreased cyclin D1 expression (28).

The proto-oncogenic role of cyclin D1 has been characterized in various studies (16-19). We propose that our immunohistochemical finding of an increased cyclin D1 expression in thin invasive melanoma also suggests a potential role of cyclin D1 during the process of invasion in the stepwise evolution of melanoma as thoroughly described by Shain et al (28, 29).

Alongside cyclin D1, genetic alterations leading to acquisition of invasive behaviour have been thoroughly investigated. Loss of the *cyclin dependent kinase inhibitor 2A* (*CDKN2A*) tumour suppressor gene is the most common acquired genetic change in invasive melanoma (5). Bastian et al described the mechanism of melanoma initiation as a result of bi-allelic loss of *CDKN2A*, and consequently $p16^{INK4A}$, via activation of BRN2, a transcription factor downstream of *CDKN2A* (30). Further investigations will be necessary to fully elucidate all aspects of invasion, thereby possibly uncovering new prognostic markers.

Inevitably, the results of our study need to be interpreted with certain limitations in mind. Matching of the two main groups (MTM and NMTM) was achieved for most characteristics, except for age, subtype and Breslow thickness. Exclusion of roughly 50% of all samples after acquisition from external laboratories for above mentioned reasons resulted in this unfortunate imbalance of subgroups regarding certain aspects. However, and most importantly, we do believe that the difference in Breslow thickness between the MTM and NMTM group does not represent a significant confounder of our main findings for two reasons. First, dermal Ki-67 expression retained its prognostic value also when Breslow thickness was taken into account in a multivariate analysis. Second, we did not detect any correlation between dermal Ki-67 expression and Breslow thickness, particularly not in the MTM subgroup in which median Breslow was increased.

In this context, discrepancy in Breslow measurement has to be addressed briefly. Our values for tumour thickness differed in a surprising number of patients when external samples were re-evaluated. This is particularly of importance if the mismeasurement leads to “understaging” of disease. These findings underline that a multi-eye principle is essential and generally advisable in melanoma histopathology.

In conclusion, we found an association of dermal Ki-67 expression with an increased risk of metastasis development in thin primary melanomas. The usefulness of Ki-67 expression as a prognostic marker should be considered in clinical practice and could be enhanced through combination with other methods. Although our findings suggest a potential role of cyclin D1 during early invasion of melanoma cells, expression of this protein alone does not appear to be of prognostic significance.

Figure 1: Illustration of increased epidermal cyclin D1 expression in thin invasive melanoma (A, B) compared to in situ melanoma (C, D). A-D: 100x magnification; A+C: H&E stain, B+D: Cyclin D1 stain.

Figure 2: Increased frequencies of dermal Ki-67 positive tumour cells were found in metastatic (A - C) compared to non-metastatic (D - F) thin melanomas ($P = 0.001$).

Supplementary Figure 1: Flow chart of sample acquisition.

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Author's contributions:

Corina Kaufmann, Peter Koelblinger and Werner Kempf performed the research.
Evelyn Lattmann inspired the study with her roundworm research.
Peter Koelblinger and Reinhard Dummer designed the research study.
Roland Lang and Michael Emberger contributed essential tissue samples.
Joanna Mangana contributed data by managing the melanoma registry and supervised Corina Kaufmann.
Andreas Kaiser assisted and partially performed statistical analysis of the data.
Corina Kaufmann and Peter Koelblinger analysed the data and wrote the paper.
Joanna Mangana, Roland Lang, Phil Cheng, Mitchell Levesque and Reinhard Dummer revised the paper.

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Table 1: Patient and tumour characteristics

Clinical data				
	Metastatic (n = 42)	Non-metastatic (n = 48)	In situ (n = 22)	*P* MTM / NMTM
AJCC Stage IV	28 (66.7%)			
AJCC Stage III	14 (33.3%)			
Female	15 (35.7%)	28 (58.3%)	11 (50%)	0.032 †
Male	27 (64.3%)	20 (41.7%)	11 (50%)	
Mean age at first diagnosis [y]	49.2 (95% CI = 44.8 - 53.7)	62.9 (95% CI = 58.2 - 67.5)	71.5 (95% CI = 65 - 78)	< 0.001 *
Breslow [mm] Mean	0.78 (95% CI = 0.72 - 0.84)	0.59 (95% CI = 0.55 - 0.63)		< 0.001 °
Median	0.80 (95% CI = 0.74 - 0.86)	0.58 (95% CI = 0.53 - 0.62)		
Histological subtype				
SSM	24 (57.1%)	20 (41.7%)	11 (50%)	invasive 0.22 †
NMM	0	2 (4.2%)	0	
ALM	3 (7.1%)	3 (6.2%)	0	
LMM	1 (2.4%)	6 (12.5%)	11 (50%)	invasive / MIS < 0.001 †
Others / unknown	14 (33.4%)	17 (35.4%)	0	
Anatomic site				
Head/Neck	7 (16.7%)	7 (14.6%)	1 (4.6%)	0.44 †
Upper extremities	4 (9.5%)	11 (22.9%)	1 (4.6%)	
Lower extremities	12 (28.6%)	15 (31.2%)	1 (4.6%)	
Trunk	17 (35.7%)	12 (25%)	2 (9%)	
Acra	4 (9.5%)	3 (6.3%)	0	
Unknown	0	0	17 (77.2%)	
Mitoses / mm²				
< 1	31 (73.8%)	39 (81.3%)		0.4 †
≥ 1	11 (26.2%)	9 (18.7%)		
Ulceration				
Present	2 (4.8%)	5 (10.4%)		0.45 †
Absent	37 (88.1%)	41 (85.4%)		
Unknown	3 (7.1%)	2 (4.2%)		
SLNB				
positive	9 (21.4%)	0		0.017 †

negative	7 (16.7%)	6 (12.5%)		
Not performed	26 (61.9%)	42 (87.5%)		
Marked Inflammation				
Present	17 (40.5%)	20 (41.7%)		
Absent	25 (59.5%)	28 (58.3%)		

Abbreviations: SSM = superficial spreading melanoma, NMM = nodular melanoma, ALM = acral lentiginous melanoma, LMM = lentigo maligna melanoma. †: X^2 test, *: t-test, °: Mann-Whitney-U-test.

Table 2: Histological data

Histological data					
	Metastatic (n = 42)	Non-metastatic (n = 48)	In situ (n = 22)	In situ vs. invasive *	Non-metastatic vs. metastatic
Cyclin D1 intensity					
None (0)	2 (4.8%)	0	0		
Weak (1)	8 (19%)	9 (18.8%)	9 (40.9%)		*P* = 0.025
Intermediate (2)	20 (47.6%)	16 (33.3%)	11 (50%)		
Strong (3)	12 (28.6%)	23 (47.9%)	2 (9.1%)		
Cyclin D1 positive cells epidermal					
0	1 (2.4%)	0	0		
1%	2 (4.8%)	1 (2.1%)	1 (4.6%)		
1 - 10%	7 (16.7%)	10 (20.8%)	14 (63.5%)		
10 - 33%	19 (45.1%)	21 (43.8%)	5 (22.7%)	*P* = 0.003	*P* = 0.611
33 - 66%	7 (16.7%)	12 (25%)	2 (9.2%)		
> 66%	4 (9.5%)	4 (8.3%)	0		
NA	2 (4.8%)	0	0		
Cyclin D1 positive cells dermal					
0	9 (21.4%)	10 (20.8%)			
1%	4 (9.5%)	6 (12.6%)			
1 - 10%	11 (26.2%)	16 (33.3%)			
10 - 33%	9 (21.4%)	10 (20.8 %)			*P* = 0.883
33 - 66%	3 (7.2%)	2 (4.2 %)			
> 66%	0	0			
NA	6 (14.3%)	4 (8.3%)			
Allred score epidermal					
None (0)	1 (2.4%)	0	0		
Weak (1 - 2)	1 (2.4%)	1 (2.1%)	1 (4.6%)		
Intermediate (3 - 5)	24 (57.2%)	22 (45.8%)	18 (81.8%)		*P* = 0.588
Strong (6 - 8)	14 (33.2%)	25 (52.1%)	3 (13.6%)		
NA	2 (4.8%)	0	0		
Allred score dermal					
None (0)	1 (2.4%)	0			
Weak (1 - 2)	11 (26.2%)	8 (16.7%)			
Intermediate (3 - 5)	17 (40.4%)	27 (56.2%)			*P* = 0.502
Strong (6 - 8)	7 (16.7%)	9 (18.8%)			
NA	6 (14.3%)	4 (8.3%)			

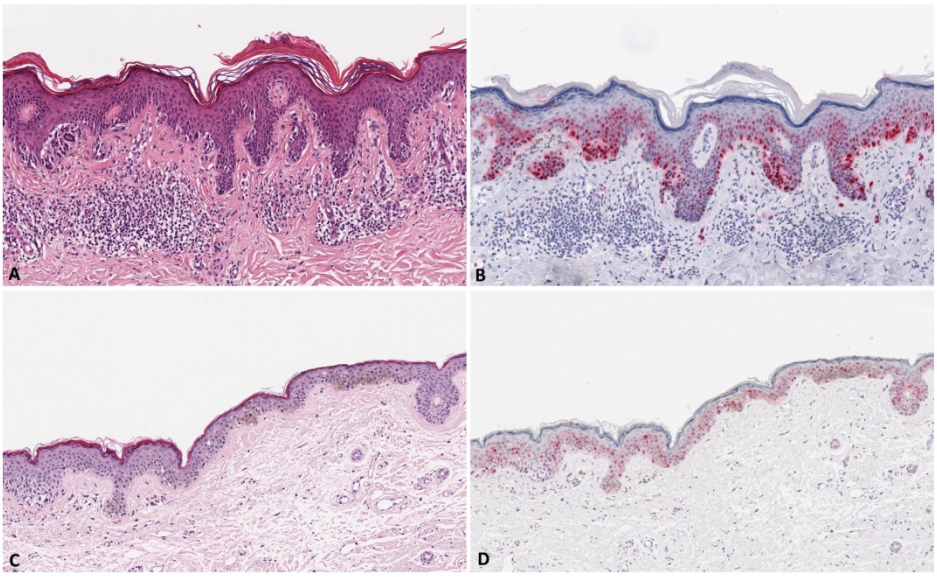
Ki-67 positive cells epidermal					
≤20%	13 (30.9%)	23 (47.9%)	10 (45.4%)	*P*=0.699	*P*=0.148
≥20%	27 (64.3%)	25 (52.1%)	12 (54.6%)		
NA	2 (4.8%)	0	0		
Ki-67 positive cells dermal					
≤20%	18 (42.8%)	38 (79.1%)			*P*=0.001
≥20%	12 (28.6%)	4 (8.3%)			
NA	12 (28.6%)	6 (12.6%)			
Ki-67+Cyclin D1 epidermal					
Low (0-4)	13 (31%)	20 (41.7%)	21 (95.4%)		*P*=0.386
High (5-7)	25 (59.5%)	28 (58.3%)	1 (4.6%)		
NA	4 (9.5)	0	0		
Ki-67+ Cyclin D1 dermal					
Low (0-4)	19 (45.2%)	38 (79.2%)			*P*=0.003
High (5-7)	8 (19.1%)	1 (2.1%)			
NA	15 (35.7%)	9 (18.7%)			

* invasive = NMTM and MTM grouped together

Table 3: Epidermal Cyclin D1 expression in different subtypes

Epidermal Cyclin D1 expression in different subtypes										
								P†		
Cyclin D1 positive cells		0	1%	1-10%	10-33%	33-66%	>66%	SSM/ LMM	SSM/ ALM	LMM/ ALM
SSM	55 (49.1%)	3 (5.5%)	2 3.6%)	5 (9.1%)	31 (56.4%)	10 (18.2%)	4 (7.3%)	0.019	0.015	0.059
LMM	18 (16.1%)	1 (5.6%)	1 (5.6%)	8 (44.4%)	5 (27.8%)	3 (16.7%)	0			
ALM	6 (5.4%)	0	1 (16.7%)	1 (16.7%)	0	2 (33.3%)	2 (33.3%)			
NM	2 (1.8%)									
Other	31 (27.7%)									

† Fisher's exact test



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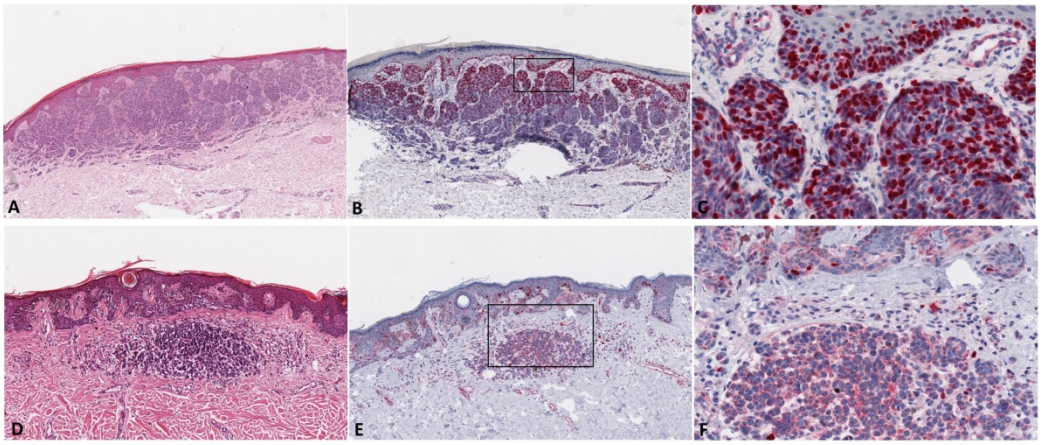

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